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Mechanisms of protein misfolding: Novel therapeutic approaches to protein-misfolding diseases

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Abstract

In protein misfolding, protein molecule acquires wrong tertiary structure, thereby induces protein misfolding diseases. Protein misfolding can occur through various mechanisms. For instance, changes in environmental conditions, oxidative stress, dominant negative mutations, error in post-translational modifications, increase in degradation rate and trafficking error. All of these factors cause protein misfolding thereby leading to diseases conditions. Both *in vitro* and *in vivo* observations suggest that partially unfolded or misfolded intermediates are particularly prone to aggregation. These partially misfolded intermediates aggregate *via* the interaction with the complementary intermediates and consequently enhance oligomers formation that grows into fibrils and proto-fibrils. The amyloid fibrils for example, accumulate in the brain and central nervous system (CNS) as amyloid deposits in the Parkinson's disease (PD), Alzheimer's disease (AD), Prion disease and Amylo lateral Sclerosis (ALS). Furthermore, tau protein shows intrinsically disorder conformation; therefore its interaction with microtubule is impaired and this protein undergoes aggregation. This is also underlying cause of Alzheimers and other neurodegenerative diseases. Treatment of such misfolding maladies is considered as one of the most important challenges of the 21st century. Currently, several treatments strategies have been and are being discovered. These therapeutic interventions partly reversed or prevented the pathological state. More recently, a new approach was discovered, which employs nanobodies that targets multisteps in fibril formation pathway that may possibly completely cure these misfolding diseases. Keeping the above views in mind in the current review, we have comprehensively discussed the different mechanisms underlying protein misfolding thereby leading to diseases conditions and their therapeutic interventions.

Keywords Protein-misfolding, Dominant-negative mutations, Amyloid, Oxidative Stress, Error in post-translational modifications, Error in trafficking, Therapeutic approaches to protein misfolding diseases

Abbreviations

A β	Amyloid beta peptide
APP	Amyloid Precursor Protein
AD	Alzheimer's disease
AGEs	Advanced Glycation End Products
ALS	AmyloLateral Sclerosis
CNS	Central nervous system
DM	Diabetes Mellitus
DBD	DNA-binding Domain
ER	Endoplasmic Reticulum
ERAD	Endoplasmic-Reticulum-Associated protein Degradation
ECMs	Extracellular Membranes
FDAP	Fluorescence Decay After Photoconversion
GAGs	Glycosaminoglycans
GSK-3	Glycogen Synthase Kinase-3
HA	Hyaluronic Acid
HS	HeparanSulfate
JNK	c-Jun N-terminal Kinase
LB	Lewy bodies
NFTs	Neurofibrillary Tangles
PD	Parkinson's Disease
PDI	Protein Disulfide Isomerase
PGs	Proteoglycans
PrP	Prion protein
PTMs	Post Translational Modifications
RAGE	Receptor for AGE
ROS	Reactive Oxygen Species

Introduction

Protein misfolding occurs because of several factors including, dominant-negative mutations, changes in environmental conditions (pH, ionic strength, temperature, and protein concentrations), error in post-translational modifications, increase degradation rate, oxidative stress and error in trafficking. Such factors may act either independently or simultaneously [1]. Various experiments (*in vitro* and *in vivo*) suggest that misfolded or partially unfolded intermediates are particularly liable to aggregation, especially at high peptide concentrations [2-6]. These partially unfolded or misfolded intermediates are enhanced under equilibrium conditions. The partially unfolded intermediates contain large patches of adjoining surface hydrophobicity, hence they can aggregate more easily than native and unfolded proteins, which possess hydrophobic amino acid situated at the interior core of protein and lie scattered in the polypeptide chain, respectively. These partially unfolded intermediates tend to aggregate by interacting with complementary intermediate and consequently enhance oligomers formation which grows into proto-fibrils and fibrils (Fig.1). The amyloid fibrils are important origins of toxicities that led to diseases conditions such as amyloidosis. The amyloid fibrils characteristically composed of 2-6 unbranched protofilaments with a diameter of 2-5 nm which is linked laterally or twisted together forming fibrils with diameters of 4-13 nm [7-9]. The fibrillar aggregates can interact with dyes such as Congo red leading to birefringence as well as thioflavin-T resulting in fluorescence.

Initial studies have shown that amyloid fibrils were the main culprit behind toxicity that led to neurodegenerative diseases. However, currently attention shifted to the cytotoxicity of amyloid fibril precursors, notably amyloid oligomers, which are the major reason of toxicity. Molecular

mechanisms that induce the formation or stabilization of oligomers of the wild-type A β remain unclear. In our earlier review [10] we have discussed that there are several mechanisms of toxicities caused by oligomers. Later on, in our review we have hypothesized two major possible mechanisms of toxicities instigated by oligomers of A β (amyloid beta), PrP (prion protein) (106-126), and α -Syn (alpha-synuclein) including direct formation of ion channels and neuron membrane disruption by the increase in membrane conductance or leakage in the presence of small globulomers to large prefibrillar assemblies. This is also validated by most recent findings that showed oligomer-related toxicities including : nonspecific perturbation of cellular and intracellular membranes and amyloid pore channel formation [11].

Intrinsically disorder conformation of tau is also important origin of different neurodegenerative diseases. Since tau protein adopts intrinsically disorder conformation therefore its interaction with microtubule is impaired and it undergoes aggregation leading to Alzheimer's diseases and several other neurodegenerative diseases. Using predictive atomic resolution descriptions of intrinsically disordered hTau40 and α -synuclein in solution from NMR and small angle scattering, enhanced polyproline II sampling occurred in aggregation-nucleation sites, supporting the suggestions that this region of conformational space plays an important role in aggregation [12]. Furthermore, majority of all prolyl bonds in the functional TauF4 fragment exist in the *trans* conformation [13]. Phosphorylation does not change this conformational state. Since, pThr231-Pro232 prolyl bond of Tau in Alzheimer's disease neurons is dominantly in the *trans* conformation, this challenges the recent concept of "cistauosis" [14]

Previous attempts have largely failed to analyze the structure of tau in complex with MTs. This is because of the mobility of the Tau structure and the dynamic nature of Tau-MT interaction. Now the molecular insights into the interaction between Tau and MTs and tau aggregation have been solved to some extent. The data showed that although intrinsically disorder tau exists as largely extended state. When bound to the MT at the interface [15, 16], it does not fold into a globular structure but rather distinct regions of Tau fold into well define hairpin structure upon binding which is formed around the PGGG motif[17]. Huang and Stultz, have demonstrated that aggregation promoting sequence or motif, PHF6*, prefers an extended conformation in both wild type and K280 mutant besides this residue K280 adopts loop turn conformation in WT MTBR2 and deletion of this residue led to an increase in locally extended conformation near the C-terminus of PHF6*. An increased propensity for extended state near terminus of PHF6* may facilitate tau aggregation. These results explain how a deletion at position 280 can promote the formation of tau aggregate [18].

Deeper insight into the tau-microtubule interactions have also been provided by FDAP analysis [19]. The data showed that tau-microtubule dynamics differ *in vitro* and *in vivo*. In particular, it was shown that diffusion of bound tau was negligible *in vivo* contrary to the findings that tau diffuses along MT lattice *in vitro* [19].

One of the important mechanisms of misfolding is the occurrence of dominant negative mutation. For instance, mutation in the insulin results in misfolding hence produce Diabetes Mellitus (DM) disease [20-23]. In the endoplasmic reticulum (ER) of pancreatic β -cells, these mutations ensue toxic misfolding in proinsulin's molecule [24, 25]. In a similar vein, changes in

the environmental conditions (temperature, pH, ionic strength and protein concentration) results in the formation of misfolded proteins which has high propensity to aggregate and cause misfolding diseases e.g. neurodegenerative diseases. Post-translational modifications error (glycation, phosphorylation, and sulfation) is another mechanism that may instigate misfolding diseases. However, phosphorylation itself is not an error, but is vital for many biological processes.

Glycation, promotes aggregation, consequently cross-linking of fibril occurs leading to diseases conditions [26]. This is validated by the findings that glycation aggravated neurotoxicity [27]. Likewise, phosphorylation promotes aggregation by forming oligomeric A β species, which act as nuclei for fibrillization [28]. These soluble small oligomers undergo conformational transition from α -helical and random coiled states to a β -sheet structure [28]. The tau protein was also shown to be peculiarly hyper-phosphorylated at different Ser/Thr residues in AD, which led to the buildup of neuro-filament sub-units in the AD brain [29,30]. Sulfation plays a crucial role in amyloid formation. It promotes formation of insoluble amyloid fibrils through aggregation of A β , which adds to the increased neurotoxicity of A β . Despite of the fact that Abetas readily self-aggregate *in vitro* forming amyloid fibrils, their interaction with proteoglycan sulfate further accelerate amyloid aggregation and fibril formation [31]. Increased protein degradation rate also directs to misfolding diseases [32]. A typical example is provided by the cystic fibrosis, which result from the removal of Phe at amino acid position 508 in cystic fibrosis transmembrane regulator protein. This mutation led the protein to misfold rendering it as a target for degradation [33]. Error in trafficking may induce dysfunction through loss of protein function at appropriate site or gain of toxic function if it accumulates at incorrect site. For instance, mutation in α -1-

antitrypsin leads to lungs diseases including emphysema because of losing the function at this site, and liver diseases by a gain of toxic function because mutant form of this protein fails to complete proper folding hence accumulate in the ER of hepatocytes leading to liver damage [34]. Similarly, oxidative stress is involved in the pathogenesis of neurodegenerative diseases such as AD, which is characterized by the deposition of intracellular aggregates that contains typically phosphorylated forms of the tau protein [35]. Thus, these mechanisms provide important therapeutic interventions. In views of above, in the current review we shall comprehensively discuss the underlying mechanisms of protein misfolding that lead to diseases conditions and their therapeutic interventions.

Protein misfolding mechanisms

When proteins such as cystic fibrosis transmembrane regulator, amyloid beta, Parkin, and Prp misfold, it gives rise to different pathological states including cystic fibrosis, Alzheimer's disease, Parkinson's disease and Prions disease respectively. The mechanism of misfolding and thereby associated diseases occur by several mechanisms, some of them are discussed below:

Changes in environmental conditions

The rate and extent of amyloid formation is greatly dependent upon the environmental conditions including changes in ionic strength, pH, temperature, and protein concentration. Recent results showed that pH affected both fibrils formation and their morphology [36]. For instance, extracellular P-(1-28) segment displayed clear pH dependence fibrils formation profile. The pH dependent fibrils formation showed that at low pH (2.5), fibrils fragments were formed with 60-80Å diameter and several hundred angstroms in length. Increasing the pH to 5.5 led to

generation of numerous large non-branched fibrils with similar diameters. These fibrils at pH 8 were mostly converted into amorphous materials, which are often found lightly stained [36]. In a similar vein, aggregation of beta amyloid protein (A β 1-42) is strongly dependent on the pH of the solution [37]. The Abeta (1-42) peptides formed large and complex fibrillar structure with higher efficiency under acidic condition than at neutral pH[37]. Furthermore, Abeta aggregates caused significant apoptotic death of PC12 cells only at pH 5.8. Thus, it is proved that the Abetas present in acidic organelles could form neurotoxic fibrils much more readily than in the neutral cellular compartments [37].

Similarly, changes in ionic strengths have profound effect on aggregation kinetics of amyloid β peptide. This was validated by molecular dynamic studies by several authors [38-40] that increase in ionic strength enhanced the atomic fluctuation of the hydrophobic core [38-40] with decline in the stability of the beta-sheet structure (which are mainly stabilized by internal hydrogen bonds). This implies that amyloid beta has high aggregation propensity at high ionic strength with strong propositions in AD.

Studies have demonstrated that temperature also has an important role in the aggregate formation [41]. For instance, at different temperatures transition of solid Abeta (1-28) and Abeta (1-40) peptides, from α -helix to beta-sheet structure, occurred near 40° C and 45°C respectively. These peptides have significant aggregation tendency. However, no transition temperature for solid Abeta (1-42) peptide occurred because it already exists as beta-sheet structure. In fact this peptide (Abeta(1-42)) does not contain alpha-helix and random coil structures but contain only beta sheet structure, hence it has high aggregation propensity. These results suggest that

temperature can also influence the A β aggregates formation which possibly may have implications in AD.

Similarly, the high concentration of A β peptide may as well affects AD. This was demonstrated by recent study that showed elevated levels of A β in CSF, which may be an index of age-related changes in the processing of the amyloid precursor protein leading to a high risk for AD [42].

Dominant negative mutations, loss of function and gain of toxic function

Dominant negative mutations are important origins of protein misfolding thereby leading to conformational diseases. For instance, mutations in the insulin gene produced DM disease [43-45, 24]. These mutations resulted in proinsulin toxic misfolding in the pancreatic β -cells [46, 47]. Mutations occur in all regions of pre-proinsulin including signal peptide and A, B, C domains. Most of these mutations initiate addition or removal of cysteine thus enhances odd number of potential pairing sites. Therefore, formation of wrong pairing of disulfide bonds occur hence causes misfolding and aggregation [46-48]. Remarkably, several human mutations encodes the similar “Akita” substitution (Cys A7 Tyr) as in the Ins2 gene of Mody4 mouse [49-51]. The variant murine proinsulin *in vitro* also goes through partial unfolding with increase formation of aggregate [52]. Similar perturbations have also been found in human insulin and proinsulin analog lacking Cys A7-B. Further, heterozygous expression of variant Ins2 allele encoding Cys A6 to Ser mutation resulted in DM.

Another instance for dominant-negative mutation is the misfolding of homotetrameric transcription factor p53 that predisposes individuals with diseases conditions [53, 54]. The p53 is a tetrameric nuclear phosphoprotein that has an important role in preventing cancer diseases [53].

The p53 enhances cell-cycle arrest or apoptosis in response to stress signals including DNA damage. Disruption of the p53 network has adverse consequences that favor cell survival and tumour progression [54-57]. The p53 is mainly regulated by the ubiquitin ligase Mdm2 (murine double mutant 2), which binds p53 and targets it for degradation by the proteasomal machinery [58]. The human p53 protein comprises 393 amino acid residues which are organized into three domains: the N-terminal activation domain interacts with various proteins; the CTD is accountable for tetramerization; and the core domain (p53C) encompassing residues 94–312 forms DNA-binding domain (DBD) [59, 60]. Over 90% of p53 dominant negative mutations are associated with diseases conditions are found in the DBD [61]. The p53 aggregation into amyloid oligomers and fibrils has already been demonstrated by Silva et al. (2014) [62]. Furthermore, the amyloid aggregates of both the mutant and WT (wild-type) forms of p53 have been identified in tumor tissues [63, 64].

Error in post-translational modifications

Normally enzymatic post translational modifications (PTMs) are highly controlled processes and play an important role in many cellular processes; however, under stressed or diseased conditions this regulation can be ineffective [65]. When enzymatic PTMs lead to excessive or differential modifications they increase the propensity of protein to form aggregate [66]. Examples of enzymatic PTMs that play crucial role in protein misfolding and aggregation are glycation, sulfation, and phosphorylation [67-70].

Glycation

Recently, great interest was shown in the role played by non-enzymatic protein glycation in inducing amyloid aggregation and toxicity. Proteins in amyloid deposits are often found glycated signifying a direct link between amyloidosis and protein glycation [70-76]. Earlier reports have demonstrated that A β is a suitable substrate for glycation resulting in the formation of advanced glycation end products (AGEs). Now accumulating evidence shows that β -amyloid (A β) is neurotoxic and its accumulation is responsible for the manifestation of Alzheimer's disease (AD). However, it is yet not clear how A β accumulates and affects toxicity. Now researchers have found that A β -AGE formation can aggravate the neurotoxicity of simple A β with up regulation of receptor for AGE (RAGE) and activation of glycogen synthase kinase-3 (GSK-3) [27]. The protein glycation has also been considered as an age related phenomenon that affects mainly extracellular proteins including collagen and elastin, which provides mechanical strength and flexibility to the tissues. AGEs formation also induce development of covalent cross-links among proteins (Fig.3). This process is gradual; therefore, cross-links stack on the long run on the oldest extracellular proteins like elastin and collagen, which are not removed by proteasomal machinery [76]. Most recently differential effect of glycation on the aggregation of proteins and amyloid formation was observed [75]. The results showed that glycation depended on both the nature of protein molecule as well as glycating agent. In fact, some glycated proteins go through oligomerization with no promotion of the amyloid fibril formation.

Glycation of α -synuclein ensues the development of toxic aggregate, which ultimately causes Parkinson's disease and Lewy bodies (LB) formation. Glycation was primarily shown to be located in substantia nigra and locus coeruleus of peripheral LB [76]. Lee *et al.* (2009) [77] have

found that methylglyoxal induces oligomerization of α -synuclein but prevents amyloid fibrils formation. Additionally, protein fibrillization was nearly completely inhibited by seeding with modified α -synuclein. Similarly, D-ribosylation of α -synuclein promotes molten globule-like aggregates formation, which, produced oxidative stress and caused high cytotoxicity to the cells [78].

Phosphorylation

Recently, studies [29] have shown that extracellular A β goes through phosphorylation by protein kinase A either as a cell surface-localized or secreted form. The phosphorylation of serine residue 8 induces oligomeric A β aggregates formation that eventually undergoes fibrillization (Fig.4). These small soluble oligomers undergo conformational transition from α -helical and random coiled states to β -sheet structure, as proved by their circular dichroism spectra [28]. The phosphorylated A β also accumulate with aging. This was established by phosphorylation-state specific antibodies that showed the occurrence of phosphorylated A β in murine AD models and in the brain of AD patient's. Remarkably, these antibodies proved that phosphorylation takes place favorably at free extracellular A β rather than at the full-length APP or β -CTF, the precursors of A β peptide. These phosphorylated A β are co-found with non-phosphorylated A β in extracellular plaques [30]. Phosphorylated A β was identified at the age of 2 months in APP transgenic mice. Consequently, phosphorylation of A β may possibly be pertinent in the pathogenesis of late onset AD. Recently, it has been shown that phosphorylation of the amyloid β -peptide at Ser26 stabilizes oligomeric assembly and increases neurotoxicity [79].

Tau is a microtubule-associated protein, responsible for the assembly and stability of microtubule in the neuronal cell and for axoplasmic transport. Tau was shown to be atypically hyperphosphorylated at several Ser/Thr residues in AD and thereby detaches from axonal microtubules and aggregates into insoluble NFT [30, 80]. However, the pathophysiological mechanism of tau phosphorylation is still a debatable issue.

Sulfation

Recently, Ariga and co-workers [31] have demonstrated that binding of A β to extracellular membranes (ECMs) is a critical step in the development of AD. A β also binds to many other biomolecules such as lipids, proteins, and proteoglycans (PGs). PGs play an important role in amyloid formation (Fig.4). It promotes aggregation of A β into insoluble amyloid fibrils that adds to the neurotoxicity of A β . Even though Abetas freely self-aggregate to yield amyloid fibrils *in vitro*, their interaction with PGs and heparan enhanced amyloid aggregation and fibril formation. The glycosaminoglycans (GAGs) sulfate moiety, the carbohydrate portion of PGs, play an important role in the amyloid fibrils formation [81]; no fibrils are formed in the presence of hyaluronic acid (HA), a non-sulfated GAG. PGs and A betas co-localize in senile plaques (SPs) and neurofibrillary tangles (NFTs) in the AD brain. The 13-16-amino-acid region (His-His-Gln-Lys) of PGs binds to Abetas and serves a distinctive target site for the prevention of the formation of amyloid fibril; His13 in particular is an important residue critical for the binding to GAGs.

Glycosaminoglycans (GAGs) are also present in PrP (Sc) deposits. *In vitro* PrP(C) misfolding was enhanced by GAGs. They are co-found in cellular compartments with PrP(C) and were

suggested to be disease modifying *in vivo*. Most recently, the effects of sulfated GAGs, heparan and heparan sulfate (HS), on disease associated misfolding of full-length recombinant PrP were studied [82]. Heparan and HS induced a β -sheet conformation in recombinant PrP that became aggregated; however, the aggregates produced in the presence of heparan or HS have different solubility and protease resistance properties. Thus, minor alterations in the physico-chemical characteristics of prion disease cofactors may initiate protein misfolding.

Proteolytic cleavage

Proteolytic cleavage also leads to protein misfolding thereby neurodegenerative diseases [66, 67]. A sequence of proteolytic cleavages occurring in the amyloid precursor protein (APP) resulted in the formation of different length of A β peptides (Fig.2). In this regard, the last cleavage is catalyzed by γ -secretase (E.C.3.4.23), which can cleave at various amino acid positions of APP including 38, 40 and 42. Of these, the A β 42 fragment showed the highest tendency to build up toxic oligomers. Such example and others suggest a role of proteolytic cleavage in triggering amyloid formation [66].

Error in degradation

Error in cellular degradation systems including ERAD or autophagy is one mechanism that raises the concentration of not only fully folded proteins but also misfolded and toxic proteins. Conversely, in addition to promoting the removal of toxic and pathology associated misfolded and aggregated proteins, activating the degradation system can results in the removal of proteins critical for the normal function and survival. Thus, improper degradation of protein can contribute to the development of more severe diseases. A typical example is provided by the

cystic fibrosis, which occurs because of deletion of Phe at amino acid position 508 in cystic fibrosis transmembrane regulator protein. This mutation triggers protein misfolding thereby it is targeted for degradation [83], thus ensues disease because of lack of this protein.

Another instance for protein misfolding disease caused by error in degradation system is provided by Gaucher's disease's which is the most widespread form of lysosomal storage diseases [84, 85]. Gaucher's disease occurs because of mutations in β -glucosidase that result in misfolding, accumulation of aggregated protein specifically in the leukocytes which are not cleared by cellular degradation machinery thus initiate toxicity and disease conditions.

Error in trafficking

For proteins to be present in particular organelles they must fold correctly. Hence, mutations that destabilize the correctly folded protein may lead to incorrect subcellular localization. This may cause dysfunction through two mechanisms including loss of protein function at appropriate site and acquisition of toxic function if it accumulates at incorrect site. The main example of this type of mutation is α -1- antitrypsin which, when mutated culminates in emphysema due to the loss of function, and liver diseases by a dominant acquisition of toxic function because the mutant protein fails to establish proper folding, hence they are retained in the ER of the hepatocytes causing liver damage [34]. Moreover, because mutated protein is not secreted into bloodstream, hence it is unable to inhibit serine protease including neutrophil elastase in the lungs leading to extensive destruction of the lung's connective tissue which culminates into emphysema.

Amyloid accumulation

Amyloid fibers are insoluble fibrous protein aggregates, which accumulate and contribute to a variety of different neurodegenerative diseases including AD, Parkinson's disease and Huntington's disease as well as amyloidoses such as familial amyloid polyneuropathy and primary systemic amyloidosis [86-88]. Currently, small oligomers are believed to be more responsible for disrupting cellular function. On the contrary, it has been proposed that amyloid deposits could have protective function because it can sequester these toxic species [89]. However, it is noted that amyloid itself can spread disease from neurons to neurons perhaps causing more havoc [90-92].

ER Stress and Oxidative Stress in Neurodegenerative Diseases

Oxidative stress and protein misfolding are associated with the pathogenesis of neurodegenerative diseases [93] including AD, PD, that are distinguished by fibrillar aggregates composed of misfolded proteins [94]. At the cellular level, oxidative stress and ER stress or both may mediate neuronal death or apoptosis. Upregulation of ER stress and their markers have been detected in the post-mortem brain tissues and cell culture models of many neurodegenerative disorders including PD, AD, amyotrophic lateral sclerosis(ALS) and expanded polyglutamine diseases e.g. Huntington disease and spinocerebral ataxias [95]. Latest reports show that oligomeric forms of polypeptides maybe the most toxic form that caused neuronal death. However, the role of these oligomeric species in ER function and ROS generation is currently not understood.

Oxidative stress is involved in the pathogenesis of neurodegenerative diseases such as AD which is characterized by the deposition of intracellular aggregates containing abnormally

phosphorylated forms of the microtubule binding protein Tau[35]. Using a drosophila model of AD it was shown that oxidative stress plays a crucial role in neurotoxicity by promoting tau phosphorylation. Further, in such model of activation the JNK pathway correlated with the degree of tau-induced neurodegeneration [96]. Although oxidative stress and ER stress have been associated to neurodegenerative diseases but to-date it has not been possible to confirm that these processes are the principal causes of neurons death. However, these stresses have been reported to alter the evolution and severity of such complex diseases.

PD is considered as the second most common neurodegenerative disease which is distinguished by the dopaminergic neurons loss. Analysis of familial Parkinson disease revealed involvement of three genes encoding α -synuclein, Parkin and ubiquitin C-terminal esterase L1 (UCH-L1). α -synuclein is a cytoplasmic protein that forms aggregate called Lewy bodies that are characteristic for Parkinson's disease; however, the link between α -synuclein and ER stress is currently not understood. Parkin is an ubiquitin protein ligase (E3) (EC 6.3.2.19) involved in ERAD [96]. However, the expression of parkin is induced by ER stress [96]. These observations suggest the involvement of ER stress in PD. Additionally; several more studies suggest the relation between ER stress and PD. Firstly, PD mimetics e.g. 6-hydroxydopamine specifically prompt ER stress in neuronal cells [97]. Secondly, expression of ER chaperones is upregulated in the brain of PD patients and PDI is accumulated in Lewy bodies [98]. The observation of PDIp, a homologue of PDI, in experimental PD and Lewy bodies suggest that oxidative protein folding in ER may possibly be disturbed in PD.

Therapeutic strategies to protein misfolding diseases

Numerous sporadic and genetic diseases occur mainly due to protein misfolding. To treat these devastating disorders is a great challenge. Some of the therapeutic interventions are described below.

Manipulation of environmental factors for regulating protein misfolding diseases

By modulating environmental conditions, aggregation of amyloid beta protein and their associated diseases can be avoided. For instance alkaline pHs are most far away from the PI of Abeta, which means that the average deprotonation state of carboxylates is greatest relative to the average protonation states of amines or imines at low pH. Therefore, charge repulsion would be expected to interfere with intra- and inter-molecular interactions and thereby escort monomeric protein to fold and assemble [99]. Similarly, a rise in the ionic strength increase the atomic fluctuation of the hydrophobic core of beta-sheet thereby it decreases the amount of Abeta aggregate. Further, elevated temperature (such as fever) can also give rise to structural alterations in A β (tangles and plaques) or change the brain characteristic identical to those observed in AD[100]. This study provides important therapeutic interventions.

Researchers have discovered various proteins called secretases: BACE-1(EC 3.4.23.46), BACE-2(EC 3.4.23.45) and gamma secretase (E.C.3.4.23) [101-105] are involved in cutting APP into beta-amyloid. Changing the cutting behavior of such proteins may inhibit or reduce beta-amyloid development. A class of therapeutics named “secretase inhibitors” can inhibit the cutting action of secretases. An example of these drugs in phase III clinical trials is LY-450139, a γ -secretase inhibitor (Table 1). It has proved to reduce beta-amyloid concentration of the CNS in a dose-

dependent way [106]. Similarly, a new BACE inhibitor NB-360 demonstrated greater pharmacological efficacy and strong decline in amyloid- β level and neuro-inflammation in APP transgenic mice [107].

Another, new class of drugs that reduces γ -secretase production is N-[N-(3, 5-difluorophenacetyl)-L-alanyl]-S-phenyl glycine ester. This compound when injected into mice transgenic for human APPv717F, it decreases brain levels of A β in a dose-dependent way within 3 hour [108]. Similarly, certain NSAID analogues favorably prevent the formation of Abeta (42) over Abeta (40) and do not disturb Notch processing [109] (Table 1).

Enhancing the beta-amyloid removal from the brain is another way that restores the normal levels of A β . This methodology involves mobilization of immune system for creating antibodies, which attack beta-amyloid. Similarly, administration of laboratory-made antibodies to beta-amyloid; and natural products with anti-amyloid actions also re-establish normal levels of A β .

Inhibition of Dominant negative mutations-

Dominant negative mutations results in toxic misfolding of proinsulin of pancreatic β -cells thereby leading to diabetes mellitus [46, 47]. Recently, oral sulfonylurea drugs (Table 1) were reported to treat HCNJ11 or ABCC8 mutations, thus obviating the requirement for multiple insulin injections and intensive blood glucose monitoring while improving glycemic control and hopefully preventing or delaying long-standing complications [110-112].

Small molecule inhibitors, such as Nutlins have undergone clinical trials for testing drug efficacy against cancers diseases. These compounds prevented MDM2 from binding to and stimulating WT p53 degradation thereby raising the likelihood of forming WT, functional tetramers [113]. Since p53 is involved in various forms of cancer, different compounds that restored the function of mutant p53 have been synthesized. The mechanism by which most of these compounds exert their actions is currently not known; however for one compound, pk7088, the mechanism is well understood [114]. This compound interacts with and stabilizes p53 mutant, Y220C, restores normal functions similar to that of WT protein [114].

Inhibition of Post-translational modifications

Post-translational alterations of proteins such as glycation, sulfation, and phosphorylation promote misfolding, oligomerization and fibril formation. Hence, inhibition of these post-translational modifications could potentially control the course of protein misfolding diseases.

Inhibition of glycation

Recently, it has been demonstrated that glycation aggravated neurotoxicity of A β with upregulation of receptor for AGE (RAGE) and activation of glycogen synthase kinase-3 (GSK-3)[27]. Glycation was inhibited by concurrent application of RAGE antibody or GSK-3 inhibitor, which cured the neuronal damages exacerbated by glycated A β . Similarly, A β is also glycated with age-dependent elevation of AGEs in Tg2576 mice that bring about cognitive deficit in mice. The A β -AGE formation was blocked by subcutaneous infusion of aminoguanidine for 3 months. The result showed that early cognitive deficit in mice was significantly reversed [27].

Tenilsetam (CAS 997: (+/-)-3-(2-thienyl)-2-piperazinone), a cognition-enhancing drug has been successfully used for the treatment of patients suffering from Alzheimer's disease; it prevents protein crosslinking by AGEs *in vitro* [115]. The mechanism of Tenilsetam action involves covalent attachment to glycated proteins, thus blocks the reactive sites for further polymerization reactions.

Similarly, plant derived poly-phenols can also provide therapeutic alternatives to hinder the development of AGEs and RAGE-mediated neuro-inflammatory diseases, including Alzheimer's disease [116]. For example, curcumin and resveratrol possess the potential to inhibit AD due to their anti-amyloidogenic, anti-oxidative and anti-inflammatory properties [117]. Furthermore, naturally occurring compounds such as (-) epigallocatechingallate (EGCG) may as well exhibit protective effects against AGE-induced injury of neuronal cells *via* its antioxidative characteristics, and by inhibiting AGE and RAGE mediated pathways, proposing a valuable role of tea catechin against neurodegenerative diseases[118].

Carnosine a natural dipeptide that has been discovered at elevated levels in brain tissue and the innervated muscle of humans. It has strong antioxidant, metal chelating and antiglycating properties. Carnosine protects neurotoxicity caused by glycated β -amyloid peptide (A β 25-35) to rat brain vascular endothelial cells (RBE4 cell). The homologs of carnosine such as β -alanine and homocarnosine can also act as therapeutic agents but these drugs are not as effective as carnosine. Thus, it is suggested that carnosine serves as an antiglycating and antioxidant agent that protect RBE4 cells from Alzheimer's disease [119].

Inhibitor of phosphorylation

Tau protein in Alzheimer's disease becomes hyperphosphorylated that may add to neuronal degeneration. However, the involved protein kinases are still unknown. Recently, lithium (a glycogen synthase kinase-3 inhibitor) was found to produce tau dephosphorylation at the recognized sites by antibodies of Tau-1 and PHF-1 both in cultured neurons and *in vivo* in rat brain. Lithium also inhibits the Alzheimer's disease-like proline-directed hyperphosphorylation of tau protein. Thus, lithium could be used to block tau hyperphosphorylation in AD [120]. Similarly, substituted propanone also inhibits the development of abnormally phosphorylated paired helical filament epitope [121]. Chiron company has manufactured purine derivatives which inhibit GSK-3 activity [122].

The bisindolylmaleimides GF 109203x and Ro 31-8220 are shown to be potent inhibitors of GSK-3 [123]. Recently, SmithKline Beecham Company has synthesized novel aminoarylmaleimide derivatives which potently inhibited GSK-3 activity and is therefore useful for the treatment of AD, depression, cancer and non-insulin dependent diabetes [124].

Similarly, indenopyrrolocarbazole derivative is an effective modulator of multiple classes of protein kinase. This compound showed neuroprotective effect in three different animal models of motor neuron degeneration [125].

Simple heterocyclic compound such as hydroxyflavones or pyrimidones are observed to be GSK-3 inhibitor [126,127]. Therefore, compound which inhibits GSK-3 action may demolish A β -amyloid protein neurotoxicity and the development of paired helical filaments.

Further, ligands of the small molecule p75NTR decrease pathological phosphorylation and misfolding of tau, inflammatory alterations, cholinergic degeneration, and cognitive deficits in A β PP (L/S) transgenic mice [128].

Inhibitor of sulfation

Glycopolymers carrying sulfated saccharides were reported to block the formation of amyloid fibrils. Circular dichroism spectral studies demonstrated the dependence of the amyloid β peptides conformation on glycopolymer additives. These additives reduced beta-sheet contents. This was established by neutralization activity conducted by *in vitro* examination in HeLa cells. Both sulfate group and sugar moiety were observed to be important for the inhibition [129].

Similar studies on the sulfated glycans effect on PrP metabolism in scrapie-infected neuroblastoma cells were performed [130]. The results showed that pentosan polysulfate, like amyloid-binding dye Congo red, prevented the accumulation of PrP-res in the cells with no obvious effects on normal isoform metabolism. The inhibition prevented new PrP-res accumulation instead of destabilization of pre-existing PrP-res. Further, PrP-res accumulation remained declined in the cultures even after removing the inhibitors. The activities of other sulfated glycans *in vitro* show that PS, lambda-carrageenan, and dextran sulfate 500 being highly more potent as blockers of PrP-res accumulation than heparan or chondroitin sulfate. Since, the

PrP-res amyloid is identified to encompass endogenous sulfated glycosaminoglycans, in this scenario these compounds may compete for blocking the interaction between PrP and endogenous glycosaminoglycans, thus exert potent anti-PrP-res activity in neuron-derived cells infected with scrapie thereby preventing amyloid formation. This report also demonstrated that the density of sulfation and molecular size are key factors that influence anti-PrP-res activity of sulfated glycans [130].

Inhibition of improper degradation

Association with multiple chaperones and co-chaperones is a requirement of the CFTR maturation and degradation. Disrupting the function of these chaperone systems may permit mutant CFTR to escape degradation. After knocking-down AHA1, a co-chaperone that together with HSP90 changes the CFTR maturation, CFTR $\Delta F508$ not only becomes more stabilized but partly functional [131]. AHA1 is not the only protein that binds chaperones and mediates folding of CFTR. CHIP, a co-chaperone of HSP70, helps in the ubiquitylation and later degrade mutant CFTR [132]; therefore, blockage of the CHIP function may as well permit more CFTR to mature and function. Such studies propose that blocking the chaperone systems can be pharmacologically helpful to people with this mutation

Inhibition of improper localization

Mutation in alpha-1-antitrypsin in lungs results in loss of function and their accumulation in liver results in gain of toxic function; which can lead to lungs and liver diseases, respectively. The lungs diseases can be controlled by enzyme replacement therapy [133]. However, liver accumulation is harder to control. However, some progress has been achieved in this direction.

Since aggregates accumulated in liver could be cleared by macroautophagy. Therefore, medications that induce autophagy such as rapamycin and carbamazepine improved α -1-antitrypsin induced hepatic toxicity [134]. Alternatively, the mutant α -1-antitrypsin aggregates can be directly inhibited by drugs [135,136].

Inhibition of amyloid accumulation

Because amyloid and other pre-amyloid conformers accumulate in several diseases and share common structural features with fibril formation, intensive research has focused on creating therapeutics that generally target amyloid folds, contrary to targeting specific proteins. Indeed, oligomer-specific antibody that prevents the toxicity of several types of oligomers or recognize both amyloid fibrils and toxic oligomers, *in vitro* have been recently developed [137,138]. Recently, tau monoclonal antibody was generated based on humanized yeast models. The results showed it had impact on tau oligomerization which is indicative that these antibodies hold great promise in diagnostic of AD [139].

Most recently, it was shown that nanobodies from different origins (immune, non-immune or synthetic libraries) could inhibit individual species formed on the pathway of fibril formation [140]. Their binding to specific targets can block fibril formation at various stages ranging from first step (*i.e.* native state stabilization thereby preventing the formation of amyloidogenic intermediate) to the self-association of protofibrils [140]. Thus, these nanobodies in future may prove valuable therapeutic agent in completely curing neurodegenerative diseases. Similarly, Graphene oxide (GO) was reported to be an efficient modulator that may significantly control the amyloidosis of A β [141]. Further, an optimum combination of peony root and ginger

strongly prevents amyloid- β accumulation and amyloid- β -mediated pathology in A β PP/PS1 double-transgenic mice [142]. Now research work is focused on emerging antibodies that distinguish both conformation and sequence, thus possibly allowing for more specific therapeutics for treating neurodegenerative diseases [143]. Similarly, many small molecules have been identified that can prevent aggregate formation [144] or enhance their degradation [145].

Inhibition of Oxidative stress

Antioxidants are exogenous or endogenous compounds that prevent oxidative stress (OS). They neutralize reactive oxygen species (ROS) and other kinds of free radicals that results from oxidative stress and thus are powerful therapeutic agents. Natural antioxidants e.g. flavonoids and phenolic compounds, lipoic acid (thioctic acid), ubiquinone and idebenone, β -carotene and vitamin C are important therapeutic agents that keep all our vital organs free from OS [102].

Inhibition of upstream of oxidative stress

There are several reports that indicate neurodegenerations can be ameliorated by dietary intake or supplementary administration of natural antioxidants. Dietary intake containing a variety of antioxidants including vitamin supplements plays an essential role in preventing different neurological disorders [146].

These natural antioxidants prevented oxidation of proteins, lipid peroxidations and production of ROS thus act as an upstream of oxidative stress. An important upstream therapeutic strategy for inhibiting oxidative stress is vaccination against toxic fibril which is common to all different types of neuronal disorders. For instance, amyloid- β vaccination prevents formation of plaque

and neuron inflammation that occurs in AD [147]. This finding could provide platform for other types of neurological disorders caused by oxidative disorder.

Inhibition of downstream of oxidative stress

ROS are generated by several pathways that results in a number of side reactions, which interact with neuronal cells in a direct or indirect way. This post-oxidative stress may be prevented by natural and synthetic antioxidants. Among all antioxidants, Ginkgo biloba (EGb 761), a Chinese herb proves an excellent antioxidant that ameliorates β -amyloid induced toxicity after plaque formation [148]. In mild AD patients, this drug EGb 761 improves cognitive decline and neuronal function but in severe AD, neuroprotective role of EGb 761 is reduced. Since inflammatory reactions are common to all types of neuronal disorders. Therefore, NSAIDS are most effective downstream therapeutics that reduces inflammatory infiltration of macrophages. These drugs act *via* antioxidative mechanism, which reduces inflammatory reactions resulted from oxidative stress [149]. Similarly CPI-1189, a nitron related compound down-regulated the pro-inflammatory cytokine cascade of genes in primary glial cells. The nitron and related compounds are under phase III clinical trial [150]. A new methodology involves stimulation of *in vivo* proteins and growth factors such as brain derived neurotrophic factors, responsible for boosting memory and cognitive function in OS [151]. This methodology could particularly prove useful for treating deteriorating neurons.

Similarly, a chemical substance such as hormone estrogen (estradiol) resembles most to vitamin E in chemical structure. It contains a phenolic free radical scavenging site and thus yielding the antioxidant activity [152].

Conclusions

Protein misfolding is a process by which protein molecule acquires wrong tertiary structure thus induces protein misfolding diseases. Protein misfolding leading to diseases conditions occur by several mechanisms as discussed above. Treatment of misfolding maladies is utmost important. Several novel therapeutic strategies were discussed here. These therapeutic interventions partly reversed or prevented the pathological state. Most recently nanobodies from different origins (immune, non-immune or synthetic libraries) have been discovered. These nanobodies could target individual species formed on the pathway of fibril formation [140], thus possibly may allow for more complete treatment of neurodegenerative diseases. Now research work is focused on emerging antibodies that distinguish both conformation and sequence, thus possibly allowing more specific therapeutics for neurodegenerative diseases [143].

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Legends to Figure

Fig.1 Energy landscape scheme of protein folding and aggregation. The landscape scheme shows the different types of aggregates including amorphous aggregates, oligomers, and fibrils.

Fig.2 Proteolytic processing of amyloid precursor protein by different secretases including α -secretase, β -secretase and γ -secretase.

Fig.3 AGE synthesis, a non-enzymatic condensation reaction occurs between α -amino or N-terminal group of a protein, and the carbonyl group of a reducing sugar leading to production of cross-linked AGEs which forms fibril structure culminating into amyloid diseases.

Fig.4 Sulfation or phosphorylation of protein and their aberrant interactions give rise to fibril structure which induces neurotoxicity.

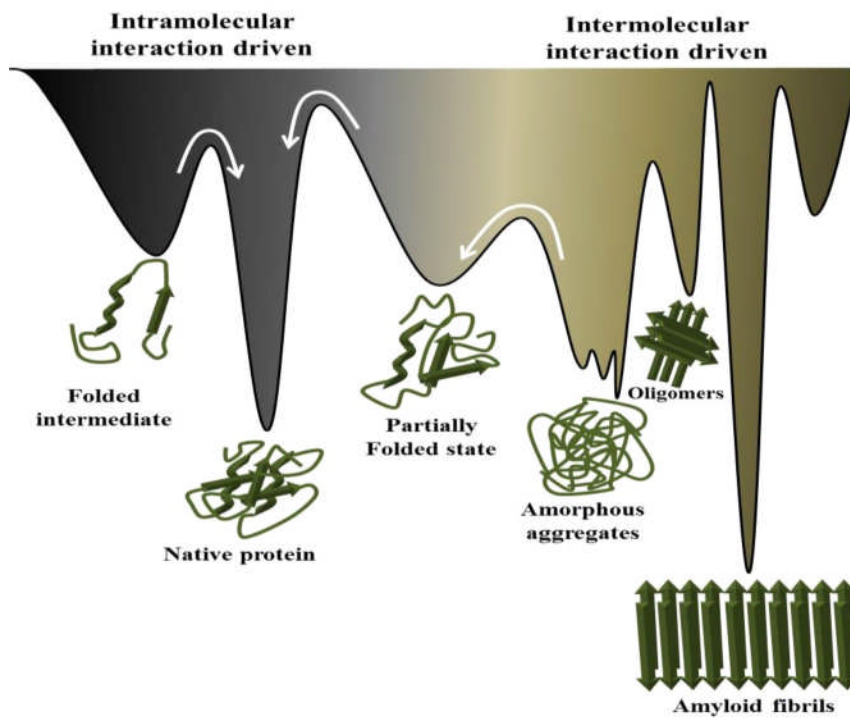


Figure 1

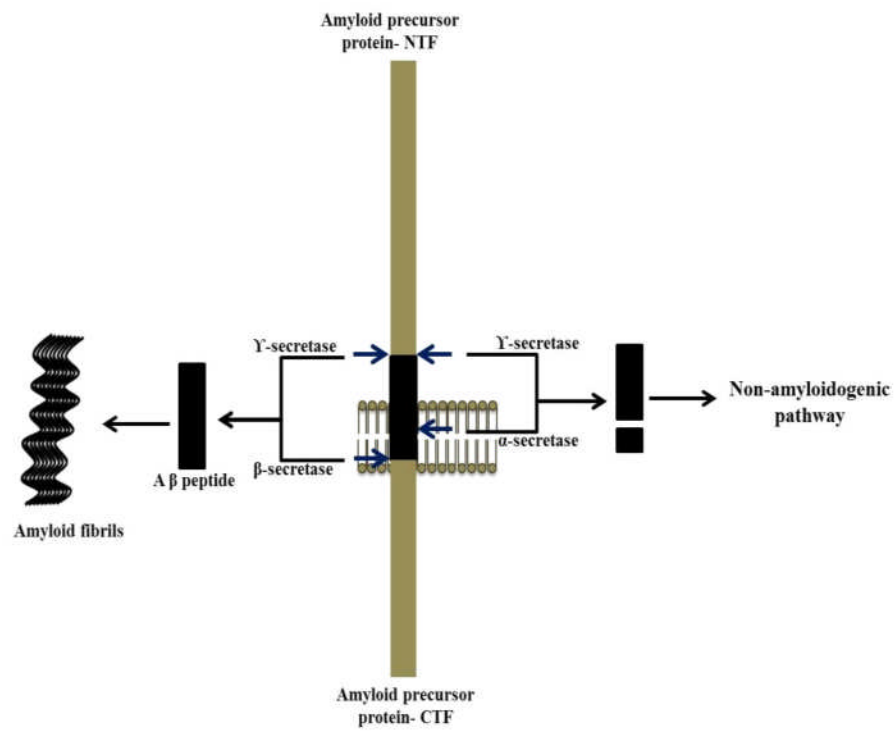


Figure 2

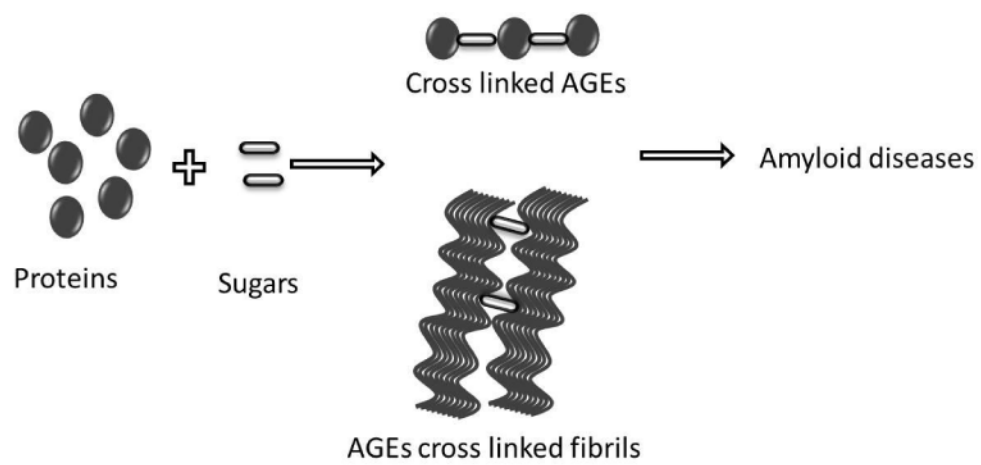


Figure 3

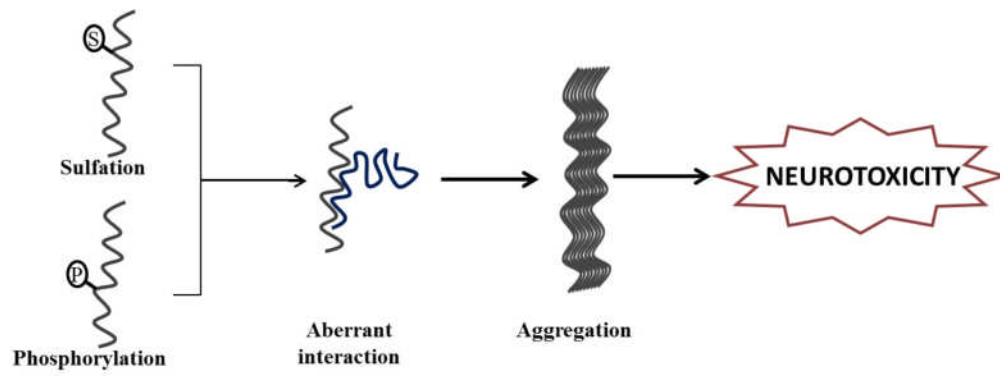
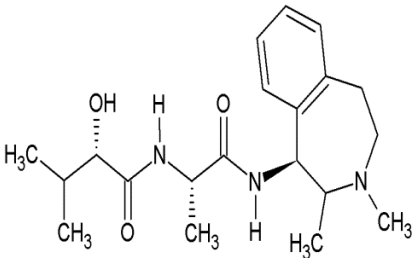
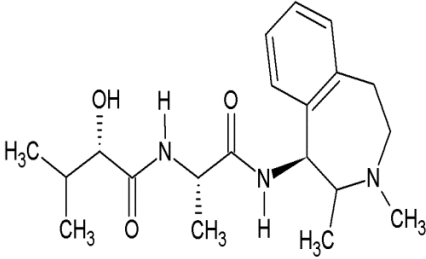
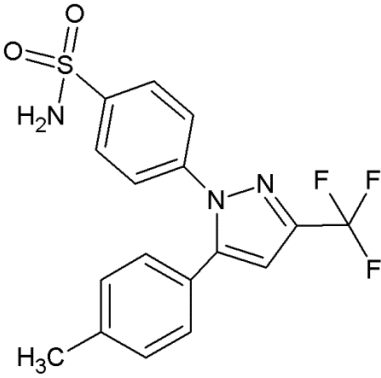
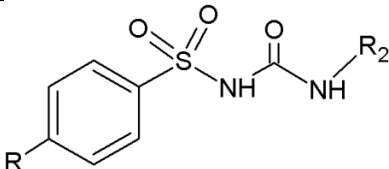


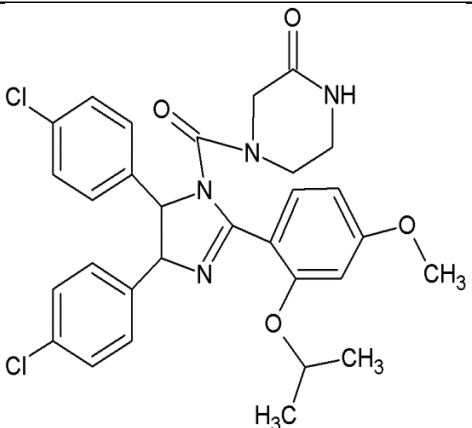
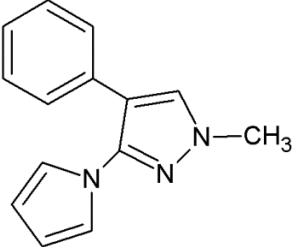
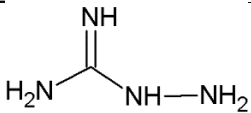
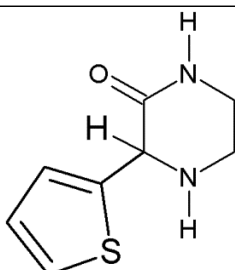
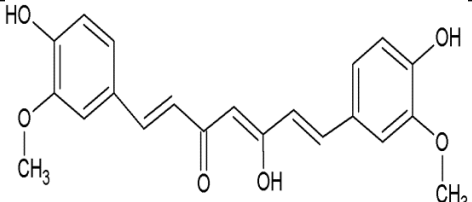
Figure 4

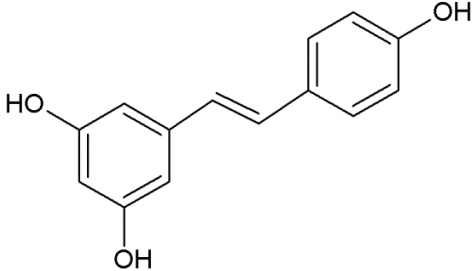
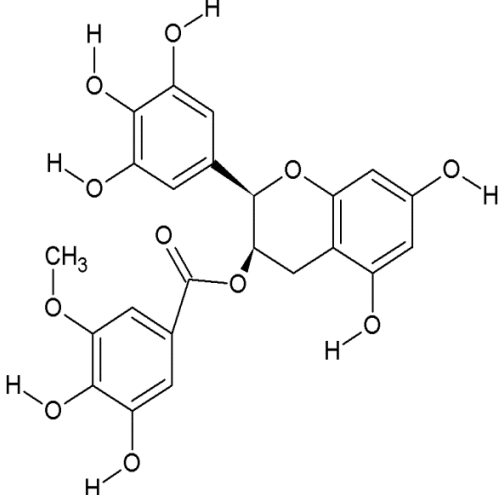
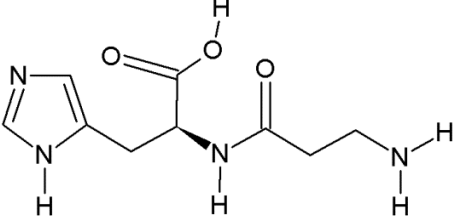
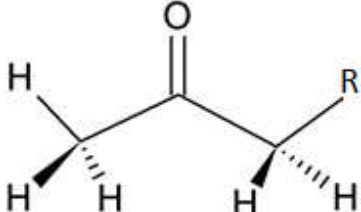
Highlights

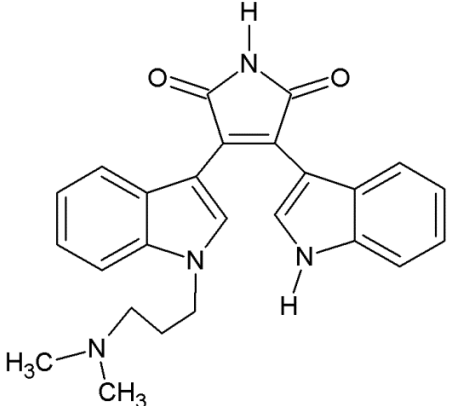
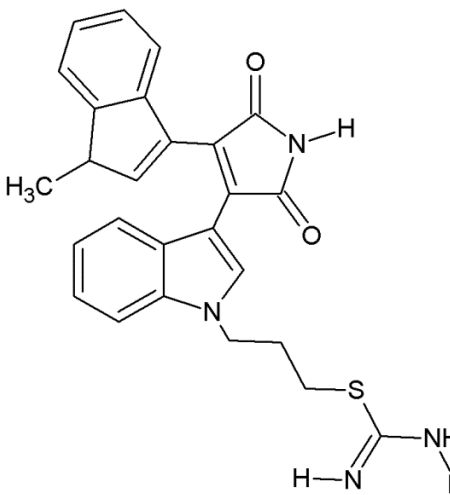
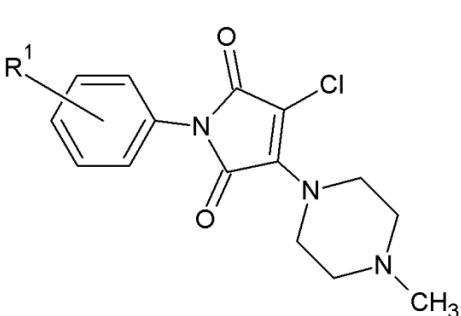
- In protein misfolding , protein molecules acquire wrong tertiary structure thereby promoting aggregation that lead to many protein misfolding diseases.
- Protein misfolding occurs because of changes in environmental conditions, dominant negative mutations,error in post-translational modifications,error in degradation, oxidative stress, and trafficking error.
- Several novel therapeutic approaches have been in vented that partly cured or reversed the pathological state.
- A novel therapeutic approach employs nanobodies that targets multisteps in fibril formation pathway thus may possibly completely cure these misfolding diseases.

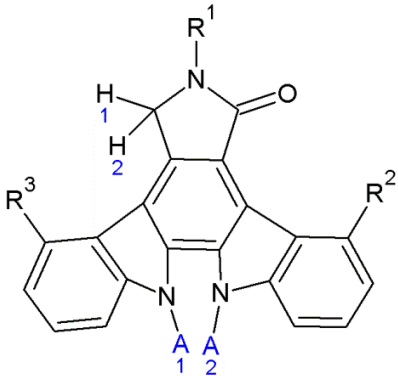
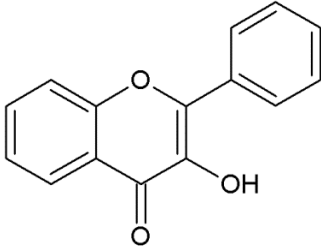
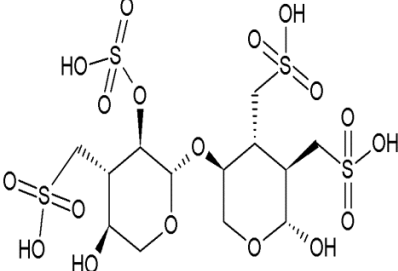
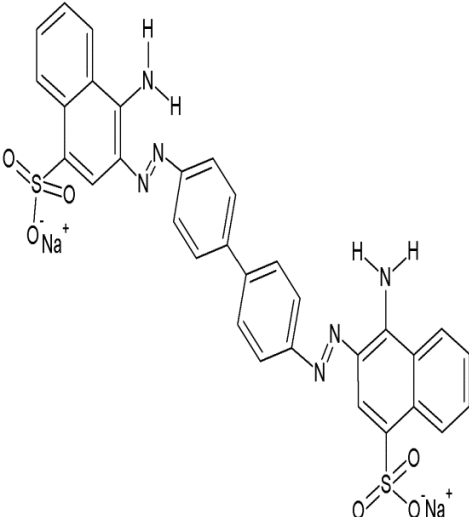
Table. 1 Structures of different drugs for treating misfolding diseases

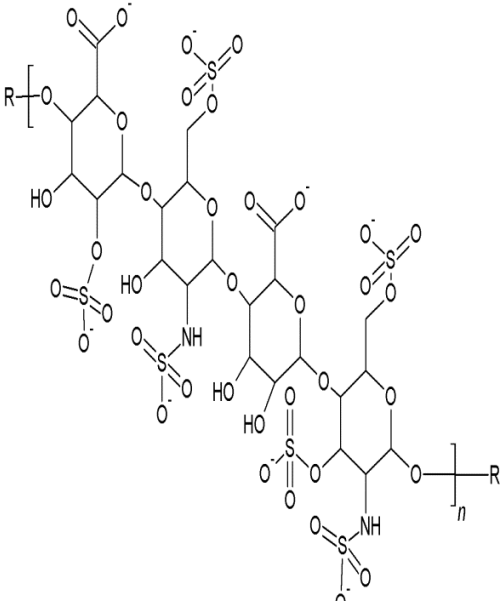
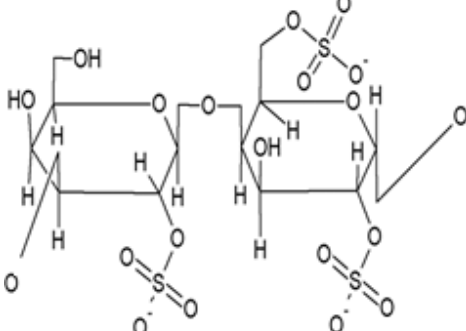
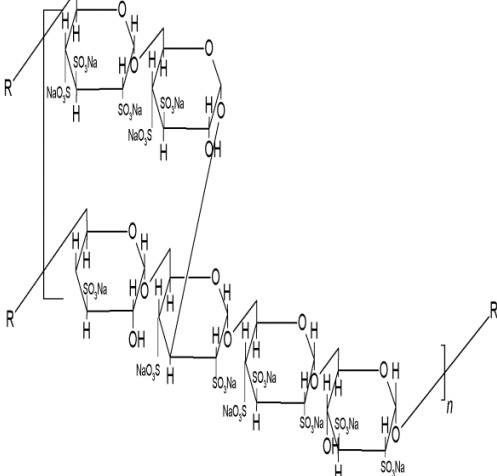
Compounds	Structures	Proteins or Compounds	References
LY-450139		γ -secretase	106
NB-360		γ -secretase	107
Celecoxib (NSAID analogue)		A β	109
Oral sulfonylurea drugs		Proinsulin	110-112

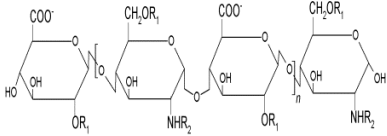
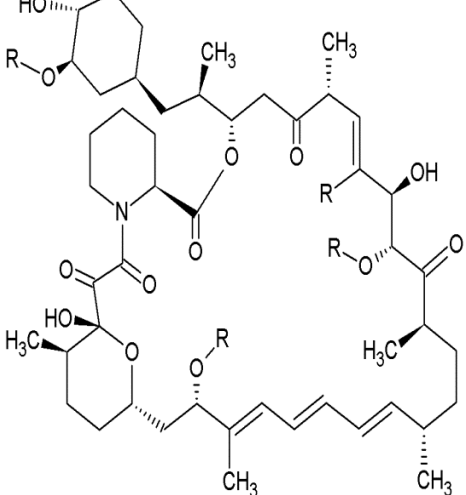
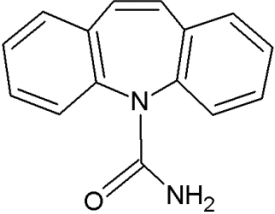
Nutlin		p53	113
pk7088		p53	114
Aminogaunidine		A β	27
Tenilsetam		A β	115
Curcumin		A β	117

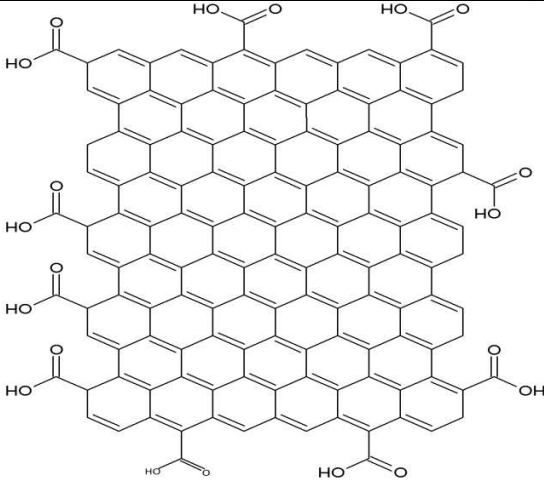
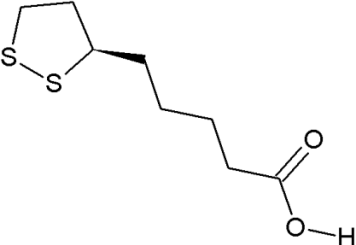
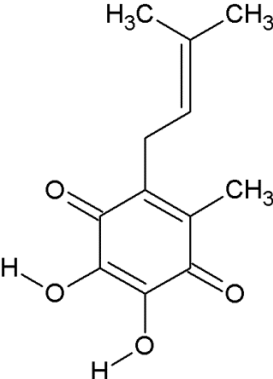
Resveratrol			117
(-)epigallocatechin gallate (EGCG)		A β	118
Carnosine		A β	119
Substituted propanone		Tau-1	121

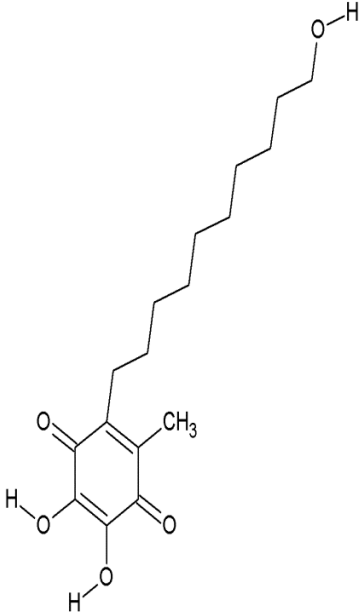
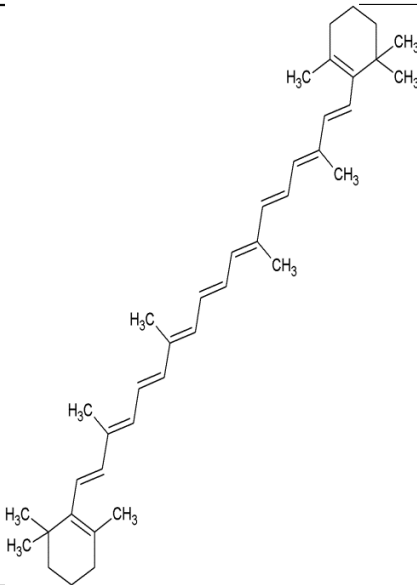
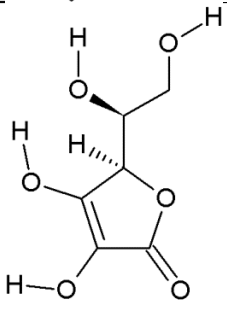
Bisindolylmaleimides GF 109203x		Glycogen synthase kinase-3	123
Ro 31-8220		Glycogen synthase kinase-3	123
Amino aryl maleimide		Glycogen synthase kinase-3	124

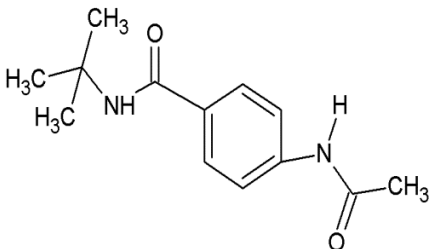
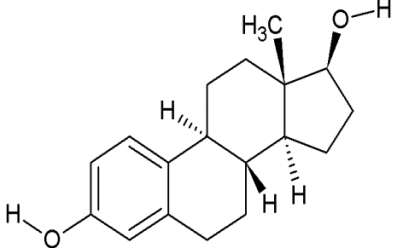
Indenopyrrolocarbazole		Protein kinases	125
Hydroxyflavons		Glycogen synthase kinase-3	126,127
Pentosan polysulfate		PrP	130
Congo red		Prp	130

<p>Heparan sulfate</p>	 <p>The diagram shows a complex repeating unit of heparan sulfate. It consists of two pyranose rings linked by a 1,3-glycosidic bond. The left ring is a glucuronic acid derivative with a carboxylate group at C5 and a sulfate group at C2. The right ring is a glucosamine derivative with a sulfate group at C2 and a carboxylate group at C5. Both rings have additional sulfate groups at various positions (C3, C6). The structure is flanked by 'R' groups and a subscript 'n' indicating it is a polymer.</p>	<p>Prp</p>	<p>130</p>
<p>Lambda-carrageenan</p>	 <p>The diagram shows a repeating unit of lambda-carrageenan, which is a disaccharide of D-glucopyranose and 3,6-anhydro-D-glucopyranose. Both sugar units are substituted with sulfate groups at the C2 and C6 positions. The structure is shown with 'R' groups at the ends, indicating it is part of a polymer chain.</p>	<p>Prp</p>	<p>130</p>
<p>Dextran sulfate</p>	 <p>The diagram shows a repeating unit of dextran sulfate, which is a polymer of alpha-D-glucopyranose. The structure shows two glucose units linked by an alpha-1,6-glycosidic bond. Each glucose unit is substituted with a sulfate group at the C2 position. The structure is flanked by 'R' groups and a subscript 'n' indicating it is a polymer.</p>	<p>Prp</p>	<p>130</p>

Sulfatedglycosaminoglycans	 <p style="text-align: center;"> $R_1 = \text{H or } -\text{SO}_3^-$ $R_2 = \text{H or } -\text{SO}_3^- \text{ or } -\text{COCH}_3$ </p>	Prp	130
Rapamycin		α 1-Antitrypsin	134
Carbamazepine		α 1-Antitrypsin	134

Graphene oxide	 <p>Aβ</p>	141	
Lipoic Acid		Reactive oxygen species(ROS)	102
Ubiquinone-1		Reactive oxygen species(ROS)	102

Idebenone	 <chem>CCCCCCCCCOc1cc(=O)c(C)c(=O)c1O</chem>	Reactive oxygen species(ROS)	102
Beta-carotene	 <chem>CC1=C(C)C(=C(C)C)C(=C(C)C)C(=C(C)C)C(=C(C)C)C(=C(C)C)C(=C(C)C)C(=C(C)C)C(=C(C)C)C1</chem>	Reactive oxygen species(ROS)	102
Ascorbic Acid	 <chem>OCC(O)C1=CC(=O)OC(C1O)O</chem>	Reactive oxygen species(ROS)	102

CPI 1189		Cytokine cascade of genes	150
Estradiol		Reactive oxygen Species	152